THE COEXISTENCE OF BICELLULAR AND TRICELLULAR POLLEN IN *ANNONA CHERIMOLA* MILL. (ANNONACEAE): IMPLICATIONS FOR POLLEN EVOLUTION[1]

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Abstract

Most angiosperms release bicellular pollen. However, in about one-third of extant angiosperms, the second pollen mitosis occurs prior to anthesis such that pollen is tricellular upon release. The shift from bicellular to tricellular development has occurred several times independently, but its causes are largely unknown. In this work, we observed the coexistence of both kinds of pollen at anther dehiscence in *Annona cherimola*, a species that belongs to the basal angiosperm family Annonaceae. Examination of pollen cell number during anther development showed that this coexistence was due to a late mitosis starting shortly prior to pollen shedding. Both types of pollen germinated equally well over the course of development. Since variable proportions of bicellular and tricellular pollen were observed at different sampling times, we tested the role of temperature by performing field and growth chamber experiments which showed that higher temperatures around anthesis advanced the time of pollen mitosis II. The results show that selection could favor the production of tricellular pollen under certain environmental circumstances that prime rapid pollen germination and provide evidence of a system in which developmental variation persists, but that can be modified by external factors such as temperature.

Key words: *Annona cherimola*, Annonaceae, bicellular pollen, cherimoya, pollen evolution, tricellular pollen

Microgametophyte development in spermatophytes is a complex process that involves an intricate interplay of different gene expression events in both the gametophytic and sporophytic tissues of the anther (for reviews see Mascarenhas, 1989; McCormick, 1993; Ma, 2005; Scott et al., 2006; Blackmore et al., 2007). In angiosperms, the sporogenous cells of the anthers differentiate to produce microsporocytes or pollen mother cells that undergo meiosis to form a tetrad of haploid microspores. Following meiosis, each unicellular microspore goes through an asymmetric mitotic division (pollen mitosis I) to produce a pollen grain with a larger vegetative cell that hosts a smaller generative cell; the latter will divide once more to produce two sperm cells (pollen mitosis II) that will be delivered into the embryo sac of the ovule during the double fertilization process typical of angiosperms to fertilize the egg and the central cell (for review see Lord and Russell, 2002). The timing of the division to yield the two sperm cells is not the same in all plant species. In most (70%) angiosperms (Brewbaker, 1967) and in all extant non-flowering seed plants (Friedman, 1999; Rudall and Bateman, 2007), pollen mitosis II takes place after pollen germination and pollen in angiosperms is therefore released from the anthers in a bicellular stage. However, in a number of unrelated angiosperm families and genera, this division occurs before pollen germination and results in pollen that is released in a tricellular stage. The ecological and evolutionary reasons behind this difference in reproductive strategy are largely unknown although tricellular pollen is known to have some common characters in different species such as a rapid germination rate and short viability (Brewbaker, 1967).

Schürhoff (1926) hypothesized that the tricellular condition could be phylogenetically derived from the bicellular condition, and Schnarf (1939) published the first summary of the taxonomic distribution of bicellular and tricellular pollen in angiosperms. Later, Brewbaker (1967) conducted an extensive survey of almost 2000 angiosperm species and not only confirmed Schürhoff's hypothesis, but revealed that the same number of pollen nuclei is

usually found within species of the same genus, and even within species of the same family or order. This hypothesis is still valid, although there are some exceptions (Webster et al., 1982; Johri et al., 1992). The tricellular condition has evolved independently from plesiomorphic bicellular pollen in several plant families, and there are no apparent reversions from tricellular to bicellular pollen (Webster et al., 1982; Soltis et al., 2005). The heterochronic shift from bicellular to tricellular pollen has occurred several times during evolution and has been associated with a selective advantage in some circumstances due to the faster germination of tricellular pollen (Brewbaker, 1967; Mulcahy and Mulcahy, 1988).

According to Brewbaker (1967), all the basal angiosperm families currently included in the eumagnoliid clade (APG II, 2003; Soltis et al, 2005) (orders Magnoliales, Laurales, Piperales and Canellales) produce bicellular pollen. Very few exceptions with tricellular pollen have been reported and include *Liriodendron tulipifera* in the Magnoliaceae, and several species of the Calycanthaceae and Monimiaceae (Johri et al., 1992). Thus, the presence of tricellular pollen reported in *Annona cherimola* Mill. (Rosell et al., 1999), a member of the Annonaceae in the Magnoliales, was unexpected. The Annonaceae or soursop family, which includes about 130 genera and 2300 species with a worldwide distribution (Chatrou et al., 2004), is the largest living family in the ancient lineage of the eumagnoliids. A high diversity of pollen development and formation is present in this family, which exhibits various pollen forms ranging from monads to polyads and different pollen binding mechanisms (Tsou and Fu, 2002, 2007).

Although the simultaneous presence of both bicellular and tricellular pollen at anther dehiscence is considered uncommon, it was reported by Maheshwari (1950) in some unrelated species and has subsequently been described in species of the order Laurales (Magnoliidae) (Sampson, 1969; Gardner, 1974), Olacaceae (Santalales) (Johri et al., 1992) and Euphorbiaceae (Malpighiales) (Webster and Rupert, 1973). Interestingly, a similar

observation has also been reported in the Araceae (Grayum, 1985), a family included in the monocot order Alismatales, which also forms part of the basal angiosperm lineages (Soltis et al., 2005). These few cases involving the simultaneous presence of both bicellular and tricellular pollen in a species at anther dehiscence have usually been regarded as anomalies or curiosities and they have not merited further attention. However, these observations could have an evolutionary/developmental cause that could represent a transitional stage in the developmental heterochronic shift from bicellular to tricellular pollen.

The number of nuclei in pollen may vary depending on the developmental stage or some external factors such as temperature. Temperature is known to affect various reproductive processes (Vara Prasad et al., 2000; Sato et al., 2002), the length of meiosis (Bennett, 1977) and mitosis (Klindworth and Williams, 2001), or overall pollen performance (Delph et al., 1997; Hedhly et al., 2005). To examine whether pollen developmental stage and temperature could affect the number of nuclei in pollen of plants known to release bicellular pollen, we determined the number of nuclei of *A. cherimola* pollen grains during the final stages of pollen development in the field and at different temperatures in controlled chambers. The results of this study highlight the coexistence of bicellular and tricellular pollen, and provide insight for our understanding of the heterochronic shift from bicellular to tricellular pollen.

MATERIALS AND METHODS

Plant material – Flowers of *Annona cherimola* and most species of the Annonaceae, are protogynous (Schroeder, 1971; Gottsberger, 1999). Moreover, the opening of flowers of the same genotype is synchronized and the transfer of pollen between different flowers of the same genotype is therefore hindered. The hermaphrodite flowers have a central pyramidal

gynoecium composed of up to 300 fused carpels and a basal helical androecium with up to 200 stamens, surrounded by two whorls of three petals. The flower opens in the morning in the female stage and remains in this stage up to the afternoon of the next day when, at the precise time of around 6 p.m. under the environmental conditions of Southern Spain, the flower enters the male stage: anther dehiscence takes place, the stigmas shrivel and the petals spread apart. Anther dehiscence occurs concomitantly in all stamens of a flower, and as the anthers dehisce they detach from the flower and fall over the open petals. Pollen is shed from the anthers in the form of tetrads (Walker, 1971) and the dehisced anthers stay in the flower overnight. Thus, the flower cycle from opening to anther dehiscence lasts two days. We use the term *F1* to designate the female stage of the flower on the first day, *F2* the female stage of the flower on the second day, *M1* the male stage of the flower on the second day and *M2* the male stage of the flower on the day after anther opening. The precise timing of the flower cycle, synchronized with particular hours of the day, enables precise sampling at defined hours prior to anther dehiscence.

Adult trees of three *A. cherimola* cultivars 'SM10', 'Campas' and 'Fino de Jete' located in a field cultivar collection at the EE La Mayora-CSIC, Málaga, Spain, were used for field experiments, which were performed over the course of three years during the flowering period from June to September.

Temperature treatments -- In order to study the effect of temperature on the final stages of pollen development and pollen germination, temperatures were monitored in the field for one month during the flowering season. Flowers were also subjected to different temperature regimes in growth chambers that reproduced temperature and relative humidity values similar to those recorded in the field. Relative humidity conditions also simulated daily variations and were the same (53-86%) in the different temperature treatments. Two

experiments were performed: one with whole trees, and another with detached flowers. In the first experiment, four trees of the cv. Fino de Jete were subjected to maximum temperatures of 20ºC, 25ºC and 30ºC. The timing of anther dehiscence was monitored in at least 80 flowers from each treatment (20 flowers per tree). In the second experiment, flowers with their pedicels were collected from trees of the cv. Campas in the field just before anthesis at 9:00 a.m. and put in 50 mL Falcon tubes with water, and then placed in growth chambers at 15ºC, 20ºC and 25ºC for 48 hours. In both experiments, anthers were collected at the time of anther dehiscence.

In vitro pollen germination **--** To evaluate in vitro pollen germination, the anthers from four flowers collected in the field were pooled. Flowers were collected at different time intervals ranging from pre-anthesis (32 hours before anther dehiscence) up to the M2 stage (15 hours after anther dehiscence). Anthers were also collected from flowers of trees maintained in the growth chambers, as well as flowers collected before anthesis from the field and maintained in the different temperature chambers. Pollen with anthers (Rosell et al., 1999) was sown on a medium of 8% sucrose, 200 mg/l $MgSO_47H_2O$, 250 mg/l $Ca(NO_3)_24H_2O$, 100 mg/l KNO_3 and 100 mg/l H_3BO_3 (Lora et al., 2006) and pollen germination was evaluated 24 hours later. The speed of pollen germination was evaluated in pollen developed in cut flowers maintained in the chambers at 15ºC, 20ºC and 25ºC. For this purpose pollen germination was recorded at 15, 30 and 105 min after sowing. Data were subjected to arcsine square root transformation and an ANOVA analysis was performed. Duncan's multiple range test was used to separate means ($p \leq 0.05$). Statistical analyses were performed with SPSS 12.0 statistical software (SPSS Inc., Chicago, USA).

Number of nuclei in pollen -- The number of nuclei in pollen was observed over the final stages of pollen development up to anther dehiscence and pollen germination from flowers collected in the field during the flowering period from June to July and flowers collected from the trees maintained in growth chambers. For this purpose, anthers from four flowers at each sampling time were collected from the field, pooled and cut transversely to extract live pollen. Pollen grains were stained with a 1:100 solution of 4´-6-diamidino-2-phenylindole (DAPI) (1mg/mL in water) and PBS for 5 min following a modification of the method by Ruzin (1999), and with propidium iodide (PI) (5 µg/mL in water) for 2-3 min. Pollen grains were also fixed for 24 hr in 3:1 ethanol-acetic acid and transferred to 75% ethanol for storage at 4ºC. The samples were dehydrated in an ethanol series, embedded in Technovit 7100 (Kulzer) resin and sectioned at 5 µm. Nuclei were observed with a solution of 0.25 mg/mL DAPI and 0.1 mg/mL *p*-phenylenediamine (added to reduce fading) in 0.05 M Tris (pH 7.2) for 1 hr at room temperature in a light-free environment (Williams et al., 1999). Preparations were observed under an epifluorescent Leica DM LB2 microscope with 340-380 and LP 425 filters for DAPI and 515-560 and LP 590 filters for propidium iodide. DAPI-stained material was also observed under a TCS-SP2-AOBS confocal microscope (Leica) with 340-380 and LP 425 filters and DIC objectives.

To evaluate the number of nuclei in germinating pollen, pollen was hydrated in a covered tray with wet filter paper for 60 min at room temperature. Stamens with pollen were then placed on a concave slide with the pollen germination medium described above and the addition of 1% agar, left to germinate and stored at -20ºC after 30 min and 105 min. Germinated and ungerminated pollen were stained with DAPI as described above. Data were arcsine square root transformed and an ANOVA analysis was performed. Duncan's multiple range test was used to separate means ($p \leq 0.05$). The association between the number of

nuclei and temperature was tested using Pearson correlation analysis. Statistical analyses were performed with SPSS 12.0 statistical software (SPSS Inc., Chicago, USA).

RESULTS

Final stages of pollen development -- In *A. cherimola* cv. Campas at the beginning of August at pre-anthesis and for the subsequent 30 hours during the female stage (F1 and F2 stages, Fig 1), pollen grains were bicellular (Fig 2a). However, 9-10 hours before anther dehiscence (F2 stage, Fig 1), the second mitotic division started in some, but not all, of the pollen grains, resulting in the release of a mixed population of bicellular (49%) and tricellular (51%) pollen (M1 stage, Fig 1). Pollen was shed loosely packed in groups of four, reminiscent of the original tetrad. After anther dehiscence, mitotic division continued in flowers left in the field overnight, and 14 hours later on the next morning (M2 stage, Fig 1) 77% of the pollen was tricellular (Fig 2b). These results were very similar to those obtained for a different *A. cherimola* genotype (M10) and at a different time of the flowering season (September). The second mitotic division also started 9-10 hours before anther dehiscence (F2 stage) and yielded a mixed population of bicellular (42%) and tricellular (58%) pollen grains at anther dehiscence. In both experiments mitotic divisions were not synchronized and pollen with two and three nuclei were observed within the same anther and even in the same tetrad (Fig 2c).

To evaluate the viability of bicellular and tricellular pollen, pollen germination was studied in vitro. Both bicellular and tricellular pollen germinated (Fig 2d and e). The fact that bicellular pollen also germinated was most apparent in samples taken from flowers at the female stage on day 2 (F2), where over 90% of the pollen was bicellular and over 30% of the pollen germinated. Pollen germination rates were similar at each time stage and increased during flower development (Table 1), reaching the highest value (64% in cv. Campas and

67% in cv. M10) at the time of anther dehiscence. At this time, 51% of the pollen was tricellular in cv. Campas and 58% in cv. M10. An examination of the number of nuclei in ungerminated pollen showed that pollen that did not germinate in vitro was generally bicellular (71%) although a small proportion (28%) was tricellular. Following anther dehiscence, pollen viability decreased rapidly and the germination rate of pollen from dehisced anthers left in flowers in the field overnight (M2 9h) was only 22%.

Effect of temperature during the final stages of pollen development -- The fact that variable proportions of bicellular and tricellular pollen were observed on the different sampling dates raised the question of whether temperature could play a role in this process.

During one month over the flowering season, pollen was collected in the field at anther dehiscence every other day. The proportion of tricellular pollen in cv. Campas fluctuated depending on the day from 24% to 62% and average field temperatures in this period varied slightly from 21ºC to 25ºC. However, the number of nuclei was highly correlated with the average temperature (Pearson's correlation coefficient $r = 0.70$, $P < 0.001$, $N = 19$). Relative humidity ranged from 43% to 78% and was weakly correlated with the number of nuclei in pollen (Pearson's correlation coefficient $r = 0.48$, $P = 0.034$). The time of anther dehiscence ranged from 17:20h to 18:45h and was significantly correlated with average field temperature (Pearson's correlation coefficient $r = 0.70$, $P = 0.001$), but not with the number of nuclei in pollen (Pearson's correlation coefficient $r = 0.30$ $P = 0.100$).

Temperature also had an effect on trees of cv. Fino de Jete kept in growth chambers. Anther dehiscence was delayed by one hour at 30ºC relative to that recorded at 20ºC. Temperature also affected the number of nuclei in pollen, and pollen developed at temperatures of 20ºC, 25ºC and 30ºC resulted in 6%, 25% and 45% of tricellular pollen,

respectively. In detached flowers of cv. Campas kept in water pollen developed at 15ºC, 20ºC and 25ºC yielded 4%, 10% and 63% of tricellular pollen, respectively.

While temperature had an effect on the number of nuclei, this was not accompanied by differences in pollen viability and no significant differences were observed for pollen germination with pollen developed at 15ºC, 20ºC and 25ºC (56%, 58% and 57%, respectively) (Fig 3). However, temperature on the final stages of pollen development affected the subsequent speed of pollen germination. Thus, pollen developed at 25ºC germinated significantly earlier in vitro compared to pollen developed at 20ºC or 15ºC (Fig 4). Interestingly, when examined 30 min after sowing on the germination medium, most germinated pollen at 25ºC was tricellular (76%), whereas bicellular pollen was predominant at both 20ºC (95%) and 15ºC (98%).

DISCUSSION

The coexistence of bicellular and tricellular pollen. – The results of this study show that bicellular and tricellular pollen are present in *A. cherimola* at the time of anther dehiscence, that both types of pollen are able to germinate and that the proportion of pollen that has advanced to the tricellular state is affected by the prevailing temperature during the final stages of pollen development. To date, this second mitosis has been consistently reported to occur either within the anther in tricellular pollen or upon pollen germination in bicellular pollen (Bedinger, 1992). These two types of pollen basically are indicators of when pollen dehydration occurs: either before or after pollen mitosis II. However, the situation reported here would be plausible if pollen dehydration was not completed upon anther dehiscence and pollen was maintained in a high-humidity atmosphere. Interestingly, these two premises are valid for *A. cherimola*: pollen is shed in a highly hydrated stage (Lora et al., 2006) and high

relative humidity is the norm at the time of flowering in the inter-Andean valleys of Southern Ecuador and Northern Peru, where the species is native (Van Damme et al., 2000). Thus, the boundary between bicellular and tricellular pollen could mainly depend on the timing of pollen mitosis II in relation to pollen shedding. Asynchrony in pollen development within the anther has been documented (Friedman, 1999) and our results could be an additional example of such a situation.

In *A. cherimola*, temperature modulates the proportion of bicellular and tricellular pollen at anthesis. This is clear from both field and controlled temperature experiments. This temperature-dependent phenotypic plasticity in the tricellular/bicellular pollen ratio could provide an adaptive advantage under changing environmental conditions maintaining variation in the time of pollen access to the ovule in order to ensure that some pollen tubes reach the egg cell at the appropriate developmental time. This would support the idea of the possible role played by phenotypic plasticity in evolution (Pigliucci, 2005).

Biological significance of the coexistence of bicellular and tricellular pollen -- An adaptive advantage of tricellular pollen is that, while it has a shorter longevity, it is ready for rapid germination (Brewbaker, 1967; Mulcahy and Mulcahy, 1988). In fact, in vivo pollen tube growth in species that shed bicellular pollen is biphasic: an initial period of slow autotrophic growth where callose plugs are absent is followed by a faster heterotrophic growth where pollen tubes produce callosic plugs (Mulcahy and Mulcahy, 1982) whereas tricellular pollen seems to rely on exogenous sources from the start of germination (Mulcahy and Mulcahy, 1988). This faster germination rate may prove valuable in conditions where rapid reproductive processes are needed. Knox (1984) suggested that tricellular pollen is related to the mode of pollen dispersal: wind- and water-dispersed pollen grains are often tricellular, and ready for rapid germination. A rapid pollen germination could also be valuable under slight

increases in temperature, which also accelerates female development and the whole reproductive process (Sanzol and Herrero, 2001).

The results of this study indicate that although both types of pollen are able to germinate, the tricellular pollen population shows a faster germination rate than the bicellular pollen population. Thus, the presence of both tricellular and bicellular pollen at anthesis could be considered a bet-hedging strategy to obtain better fertilization chances. A similar case has been proposed for pollen aperture polymorphism (Dajoz et al., 1991; Till-Bottraud et al., 1994; Till-Bottraud et al., 2005). In this case, there could be a trade-off between slow pollen germination and greater longevity (bicellular pollen), and fast pollen germination and short longevity (tricellular pollen). This could provide an adaptive advantage for a species such as *A. cherimola* with protogynous dichogamy. On the one hand, bicellular pollen could survive until the following day to be transferred by pollinating insects to flowers in the female stage (Gottsberger, 1989). On the other hand, tricellular pollen can germinate fast at anther dehiscence when stigmas can still be receptive for a short period of time.

Evolutionary significance of the coexistence of bicellular and tricellular pollen -- Although bicellular pollen is the most abundant in angiosperms and is found in about 70% of angiosperm species, tricellular pollen is considered as evolutionary derived from bicellular pollen and has arisen at different times during evolution in several families (Brewbaker, 1967). The presence of both bicellular and tricellular pollen grains in the same genus is very uncommon (Brewbaker, 1967), and few reports have documented the phenomenon of both types of pollen in the same plant at anther dehiscence. This phenomenon has been described mainly in species of fairly ancient orders such as Laurales (Sampson, 1969; Gardner, 1974) and Alismatales (Grayum, 1985), in species of the Olacaceae (Santalales) (Johri et al., 1992) and Euphorbiaceae (Malpighiales) (Webster and Rupert, 1973), and in conflicting reports of

species of different genera such as *Capsicum* (Lengel, 1960), *Populus* (Hamilton and Langridge, 1976) or *Diospyros* (Sugiura et al., 1998).

The presence of bicellular and tricellular pollen in the same species, even in the same tetrad, and the fact that the proportion of these two kinds of pollen is environmentally regulated provides an insight to understand the heterochronic shift from bicellular to tricellular pollen. This shift could be considered a matter of timing concerning mitosis in relation to pollen dehydration and shedding. Late pollen dehydration would lead to continuous pollen activity, including generative cell division, and hence tricellular pollen. However, an earlier dehydration would arrest pollen development in a bicellular state until after pollen rehydration on the stigma. Differences in the timing of gene expression between bicellular and tricellular pollen species have been revealed (Eady et al., 1994) and genes involved in male gametophyte development are being uncovered (Bedinger, 1992; McCormick, 1993; McCormick, 2004). Further knowledge on the timing of the expression of genes controlling pollen mitosis II and pollen dehydration will shed light on this hypothesis.

Thus, environmental conditions favoring high humidity in the environment close to pollen, together with an adequate temperature for generative cell division will favor the production of tricellular pollen. Although this hypothesis needs to be evaluated, it is interesting to note that Brewbaker (1967) suggested that tricellular pollen could be advantageous in aquatic taxa. Tricellular pollen also appears to be dispersed in a more hydrated state (Brewbaker, 1967; Knox, 1984), although this is not the case in Poaceae. While our results explain the possible origin of this phenomenon, the adaptive advantage that could explain why it has been fixed in several species is yet to be determined.

In this work, a combination of both types of pollen is reported at anthesis in *A. cherimola.* We could hypothesize that under certain environmental circumstances which prime rapid pollen germination, selection could favor the fixation of the trait that results in the

production of only tricellular pollen. The evolution of spermatophytes includes sequential key changes in microgametophytes that may be associated with major evolutionary diversifications (Rudall and Bateman, 2007, Williams 2008). The observations reported in this work could add an additional feature to the structural lability and developmental variation that marked the initial phases of angiosperm evolution (Friedman, 2006) and may help to understand the different strategies adopted by plants facing changing environments. It remains to be seen if the results obtained in this work highlight a particular situation of *A. cherimola* or represent a more widespread situation in flowering plants. Nevertheless, these observations give experimental support for the mechanisms underlying the many evolutionary shifts from bicellular to tricellular pollen and provide evidence for temperature-induced phenotypic plasticity.

LITERATURE CITED

APG II. 2003. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* 141: 399-436.

BEDINGER, P. 1992. The remarkable biology of pollen. *Plant Cell* 4: 879-887.

BENNETT, M.D. 1977. Time and duration of meiosis. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 277: 201-226.

BLACKMORE, S., A.H. WORTLEY, J.J. SKVARLA, AND J.R. ROWLEY. 2007. Pollen wall development in flowering plants. *New Phytologist* 174: 483-498.

BREWBAKER, J.L. 1967. Distribution and phylogenetic significance of binucleate and trinucleate pollen grains in Angiosperms. *American Journal of Botany* 54: 1069-1083.

CHATROU, L.W., H. RAINER, AND P.J.M. MASS. 2004. Annonaceae. *In* N. Smith., S.A. Mori, A. Henderson, D.W. Stevenson, and S.V. Heald [eds.], Flowering Plants of the Neotropics, 18-20. Princeton University Press, New Jersey, USA.

DAJOZ, I., I. TILL-BOTTRAUD, AND P.H. GOUYON. 1991. Evolution of pollen morphology. *Science* 253: 66-68.

DELPH, L.F., JOHANNSSON, M.H., AND A.G. STEPHENSON. 1997. How environmental factors affect pollen performance: Ecological and evolutionary perspectives. *Ecology* 78: 1632-1639.

EADY, C., K. LINDSEY, AND D. TWELL. 1994. Differential activation and conserved vegetative cell-specific activity of a late pollen promoter in species with bicellular and tricellular pollen. *The Plant Journal* 5: 543-550.

FRIEDMAN, W.E. 1999. Expression of the cell cycle in sperm of *Arabidopsis*: implications for understanding patterns of gametogenesis and fertilization in plants and other eukaryotes. *Development* 126: 1065-1075.

FRIEDMAN, W.E. 2006. Embryological evidence for developmental lability during early angiosperm evolution. *Nature* 441: 337-340.

GARDNER, R.O. 1974. Trinucleate Pollen in *Beilschmiedia nees* (Lauraceae). *New Zealand Journal of Botany* 12: 243-244.

GOTTSBERGER, G. 1989. Comments on flower evolution and beetle pollination in the genera *Annona* and *Rollinia* (Annonaceae). *Plant Systematics and Evolution* 167: 189-194.

GOTTSBERGER, G. 1999. Pollination and evolution in neotropical Annonaceae. *Plant Species Biology* 14: 143-152.

GRAYUM, M.H. 1985. Evolutionary and ecological significance of starch storage in pollen of the Araceae. *American Journal of Botany* 72: 1565-1577.

HAMILTON, D., AND P. LANGRIDGE. 1976. Trinucleate pollen in the genus *Populus*. *Experientia* 32: 467-468.

HEDHLY, A., J.I. HORMAZA, AND HERRERO, M. 2005. Influence of genotype-temperature interaction on pollen performance. *Journal of Evolutionary Biology* 18: 1494-1502

JOHRI, B.M., K.B. AMBEGAOKAR, AND P.S. SRIVASTAVA. 1992. Comparative embryology of angiosperms. 2 Volumes. Springer-Verlag, Berlin, Germany.

KLINDWORTH, D.L., AND N.D. WILLIAMS. 2001. Characterization of a mitotic mutant of durum wheat. *Chromosome Research* 9: 377-386.

KNOX, R.B. 1984. The pollen grain. *In* B.M. Johri [ed.], Embryology of angiosperms, 197-271. Springer-Verlag, Berlin, Germany.

LENGEL, P.A. 1960. Development of the pollen and the embryo sac in *Capsicum frutescens* L. var. Japanese variegated ornamental. *The Ohio Journal of Science* 60: 8-12.

LORA, J., M.A.P. DE OTEYZA, P. FUENTETAJA, AND J.I. HORMAZA. 2006**.** Low temperature storage and in vitro germination of cherimoya (*Annona cherimola* Mill.) pollen. *Scientia Horticulturae* 108: 91-94.

LORD, E.M., AND S.D. RUSSELL. 2002. The mechanisms of pollination and fertilization in plants. *Annual Review of Cell and Developmental Biology* 18: 81-105.

MA, H. 2005. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annual Review of Plant Biology* 56: 393-434.

MAHESHWARI, P. 1950. An introduction to the embryology of angiosperms. McGraw-Hill, New York, USA.

MASCARENHAS, J.P. 1989. The male gametophyte of flowering plants. *Plant Cell* 1: 657-664.

MCCORMICK, S. 1993. Male gametophyte development. *Plant Cell* 5: 1265-1275.

MCCORMICK, S. 2004. Control of male gametophyte development. *Plant Cell* 16: S142-S153.

MULCAHY G.B., AND D.L. MULCAHY. 1982. The two phases of growth of *Petunia hybrida* (Hort. Vilm-Andz.) pollen tubes through compatible styles. J. Palynol. 18: 1-3.

MULCAHY, G.B., AND D.L. MULCAHY. 1988. The effect of supplemented media on the growth in vitro of bi- and trinucleate pollen. *Plant Science* 55: 213-216.

PIGLIUCCI, M. 2005. Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology and Evolution* 20: 481-486.

ROSELL, P., M. HERRERO, AND V.G. SAUCO. 1999. Pollen germination of cherimoya (*Annona cherimola* Mill.). In vivo characterization and optimization of in vitro germination. *Scientia Horticulturae* 81: 251-265.

RUDALL P.J., AND BATEMAN, R.M. 2007. Developmental bases for key innovations in the seed-plant microgametophyte. *Trends in Plant Science* 12: 317-326.

RUZIN, S.S. 1999. Plant microtechnique and microscopy. Oxford University Press, New York, USA.

SAMPSON, F.B. 1969. Studies on the Monimiaceae. III. Gametophyte development of *Laurelia novae-zelandiae* A. Cunn. (Subfamily Atherospermoideae). *Australian Journal of Botany* 17: 425-439.

SANZOL, J., AND M HERRERO. 2001. The "effective pollination period" in fruit trees. *Scientia Horticulturae* 90: 1-17.

SATO, S., M.M. PEET, AND J.F. THOMAS. 2002. Determining critical pre- and post-anthesis periods and physiological processes in *Lycopersicon esculentum* Mill. exposed to moderately elevated temperatures. *Journal of Experimental Botany* 53: 1187-1195.

SCHNARF, K. 1939. Variation im Bau des Pollenkornes der Angiospermen. *Tabulae Biologicae* 17: 72-89.

SCHROEDER, C.A. 1971. Pollination of cherimoya. *California Avocado Society Yearbook* 44: 119-122.

SCHÜRHOFF, P.N. 1926. Die Zytologie der Blütenpflanzen. Ferdinand Enke Verlag, Stuttgart, Germany.

SCOTT, R.J., M. SPIELMAN, AND H.G. DICKINSON. 2006. Stamen development: primordium to pollen. *In* B.R. Jordan [ed.], The molecular biology and biotechnology of flowering. 298-331. CAB International, Wallingford, UK.

SOLTIS, D.E., P.S. SOLTIS, P.K. ENDRESS, AND CHASE, M.W. 2005. Phylogeny and evolution of angiosperms. Sinauer Associates Incorporated , Sunderland Massachusetts, USA.

SUGIURA, A., R. TAO, T. OHKUMA, AND M. TAMURA. 1998. Pollen nuclear number in four *Diospyros* species. *Hortscience* 33: 149-150.

TILL-BOTTRAUD, I., D.L. VENABLE, I. DAJOZ, AND P.H. GOUYON. 1994. Selection on pollen morphology - a game-theory model. *American Naturalist* 144: 395-411.

TILL-BOTTRAUD, I., D. JOLY, D. LACHAISE, AND R.R. SNOOK. 2005. Pollen and sperm heteromorphism: convergence across kingdoms? *Journal of Evolutionary Biology* 18: 1-18.

TSOU, C.-H., AND Y.-L. FU. 2002. Tetrad pollen formation in *Annona* (Annonaceae): proexine formation and binding mechanism. *American Journal of Botany* 89: 734-747.

TSOU, C.-H., AND Y.-L. FU. 2007. Octad pollen formation in *Cymbopetalum* (Annonaceae): the binding mechanism. *Plant Systematics and Evolution* 263: 13-23.

VAN DAMME, P., V. VAN DAMME, AND X. SCHELDEMAN. 2000. Ecology and cropping of cherimoya (*Annona cherimola* Mill.) in Latin America. New data from Ecuador. *Fruits* 55: 195-256.

VARA PRASAD, P.V., P.Q. CRAUFURD, R.J. SUMMERFIELD, AND T.R. WHEELER. 2000. Effects of short episodes of heat stress on flower production and fruit-set of groundnut (*Arachis hypogaea* L.). *Journal of Experimental Botany* 51: 777-784.

WALKER, J.W. 1971. Pollen morphology, phytogeography and phylogeny of the Annonaceae. *Contributions of the Gray Herbarium of Harvard University* 202: 1-132.

WEBSTER, G.L., AND E.A. RUPERT. 1973. Phylogenetic significance of pollen nuclear number in Euphorbiaceae. *Evolution* 27: 524-531.

WEBSTER, G.L., E. RUPERT, AND D. KOUTNIK. 1982. Systematic significance of pollen nuclear number in Euphorbiaceae, tribe Euphorbieae. *American Journal of Botany* 69: 407-415.

WILLIAMS, J.H. 2008. Novelties of the flowering plant pollen tube underlie diversification of a key life history stage. *Proceedings of the National Academy of Sciences of the United States of America* 105: 11259-11263.

WILLIAMS, J.H., W.E. FRIEDMAN, AND M.L. ARNOLD. 1999. Developmental selection within the angiosperm style: Using gamete DNA to visualize interspecific pollen competition. *Proceedings of the National Academy of Sciences of the United States of America* 96: 9201-9206.

TABLE 1. Percentage of pollen germination during the flower cycle in two *Annona cherimola* cultivars.

Cultivar	Stage of the flower, hour of the day					
	Pre-anthesis 9h	F1 14h	F1 19h	F2 9h	F2	M1 18h
Campas	6 ± 2 a	12.6 ± 5 b	27.9 ± 1 c	32.5 ± 1 c	47.6 ± 1 d	64 ± 10 e
M10	0 a	20.5 ± 11 b	39.7 ± 8 c	45 ± 13 cd	58.7 ± 11 de	67 ± 6 e

Notes: Data shown (Mean ± SD) for cv. Campas in August and cv. M10 in September. Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Duncan's multiple range test. F1: Female stage on the first day of the flower cycle. F2: Female stage on the second day of the flower cycle. M: Male stage of the flower cycle just after anther dehiscence.

Figure legends

Fig. 1. Percentage of tricellular pollen during the final stages of pollen development in *Annona cherimola* cv. Campas from pre-anthesis to the lapse of 48 hours. F1: Female stage on the first day of the flower cycle. F2: Female stage on the second day of the flower cycle. M1: Male stage on the second day of the flower cycle. M2: Male stage on the third day of the flower cycle. Anther dehiscence occurred at 17-19 hours on the second day of the cycle (arrow). Error bars represent standard deviations. Average temperatures during each stage are indicated below the horizontal axis.

Fig. 2. Number of nuclei on the first and second day of flower cycle. Bicellular pollen at pre-anthesis (A). Tricellular pollen at the male stage (B). The coexistence of bicellular and tricellular pollen at anther dehiscence (C). Pollen tubes 30 minutes after sowing in germination medium in vitro with two (D) and three (E) nuclei. The generative nucleus is shown with red and black arrows, and the vegetative nucleus with a white arrow. Bar = 40 μm

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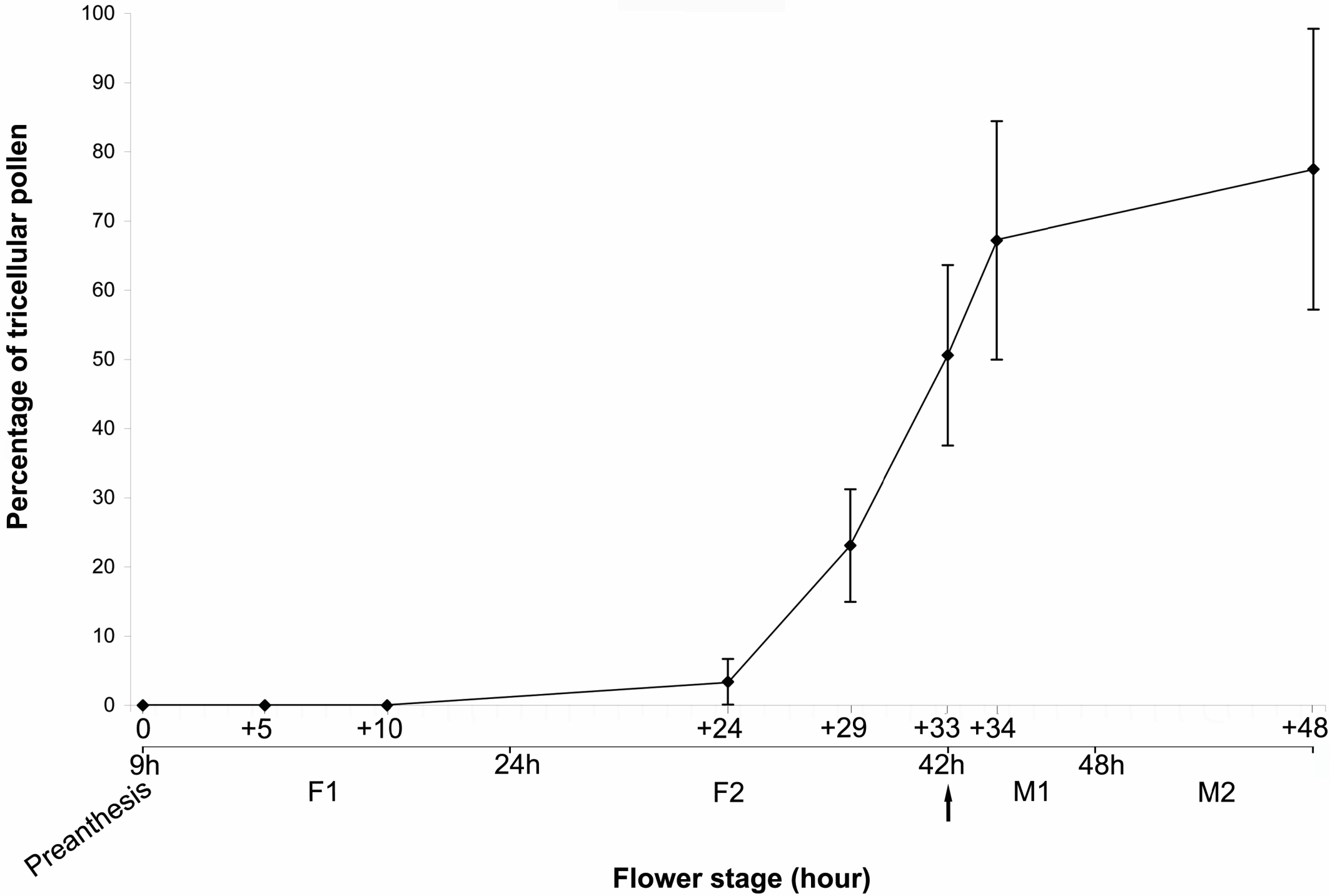
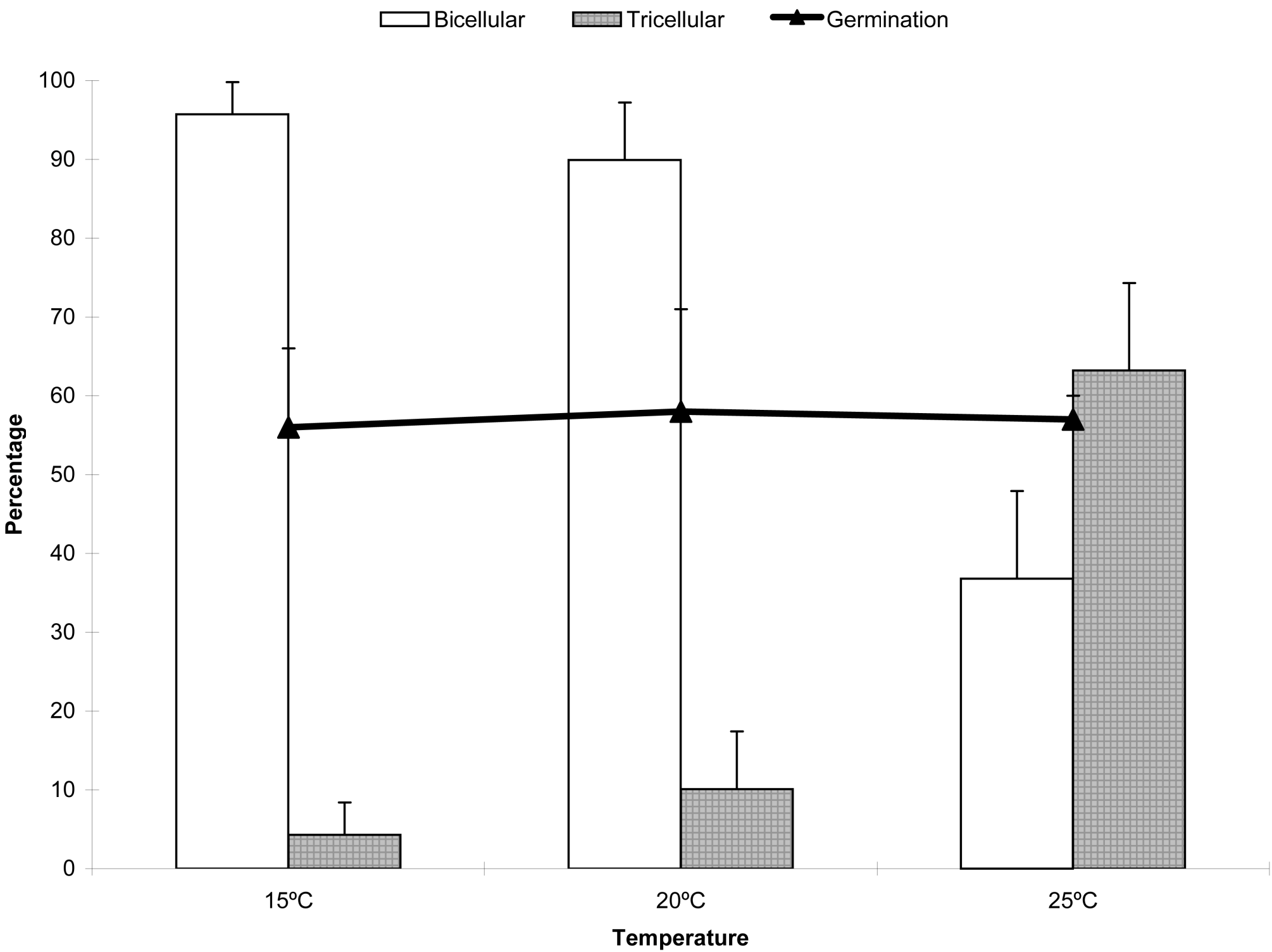
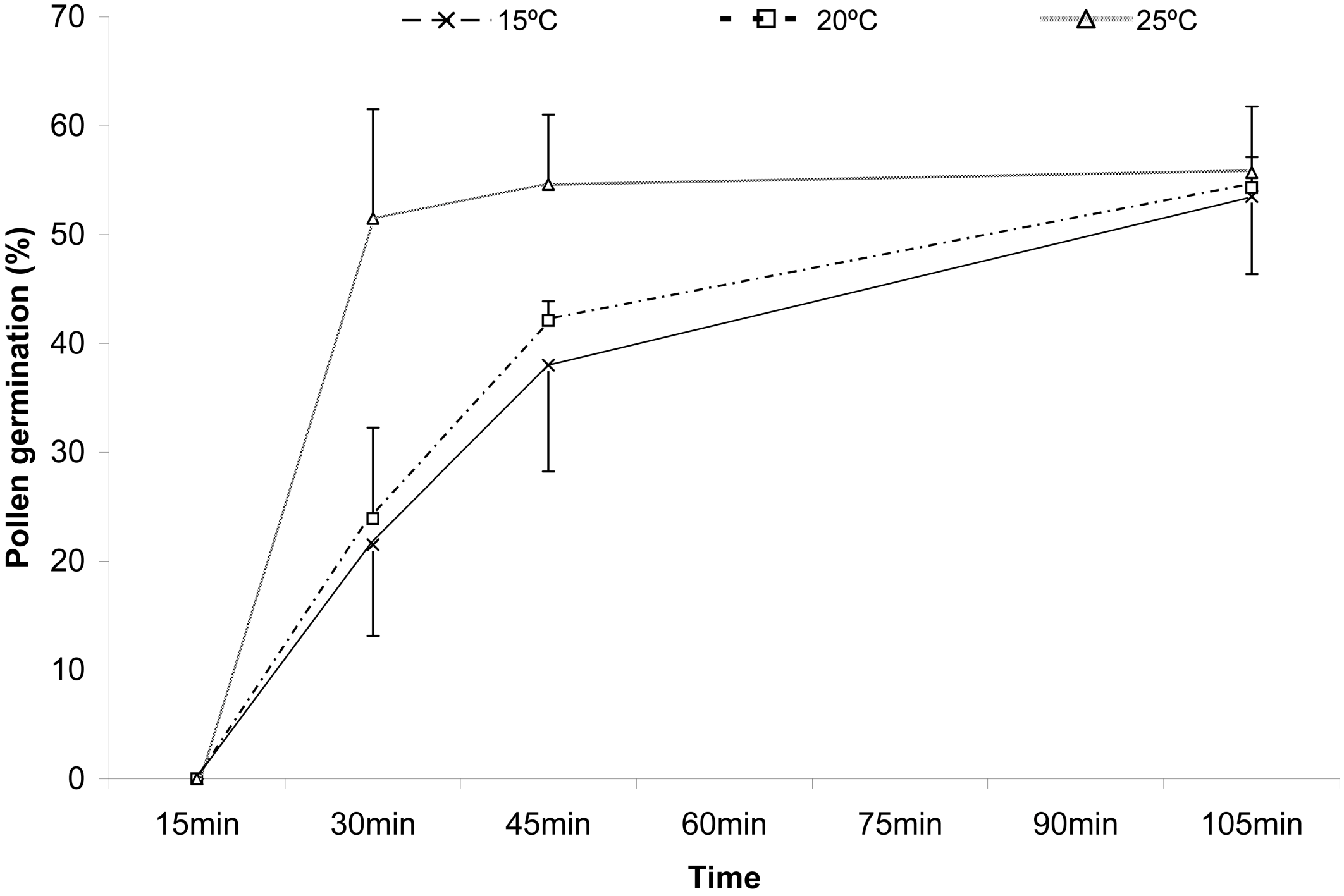
Fig. 3. Effect of temperature on pollen mitosis II and pollen germination in *Annona cherimola* cv. Campas. Bars show the percentage of bicellular and tricellular pollen at anther dehiscence from flowers kept at different temperatures during the final stages of pollen development. The black line represents the percentage of pollen germination. Error bars represent standard deviations.

Fig. 4. Pollen germination kinetics at 15, 30, 45 and 105 minutes after sowing pollen in germination medium in vitro from flowers kept at 15ºC , 20ºC and 25ºC during the final stages of pollen development in *Annona cherimola* cv Campas. Error bars represent standard deviations.


Percentage of tricellular pollen
100
90
80
70
60
50
40
30
20
10
0
0
+5
+10
+24
+29
+33
+34
+48
9h
24h
42h
48h
Preanthesis
F1
F2
M1
M2
Flower stage (hour)

A
B
C
D
E


Bicellular
Tricellular
Germination
100
90
80
70
60
50
40
30
20
10
0
Percentage
15ºC
20ºC
25ºC
Temperature


15ºC
20ºC
25ºC
70
60
50
40
30
20
10
0
Pollen germination (%)
15min
30min
45min
60min
75min
90min
105min
Time